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A Quick and Parallel Analytical Method Based on Quantum Dots Labeling for ToRCH-Related Antibodies

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Abstract Quantum dot is a special kind of nanomaterial composed of periodic groups of II–VI, III–V or IV–VI materials. Their high quantum yield, broad absorption with narrow photoluminescence spectra and high resistance to photobleaching, make them become a promising labeling substance in biological analysis. Here, we report a quick and parallel analytical method based on quantum dots for ToRCH-related antibodies including Toxoplasma gondii, Rubella virus, Cytomegalovirus and Herpes simplex virus type 1 (HSV1) and 2 (HSV2). Firstly, we fabricated the microarrays with the five kinds of ToRCH-related antigens and used CdTe quantum dots to label secondary antibody and then analyzed 100 specimens of randomly selected clinical sera from obstetric outpatients. The currently prevalent enzyme-linked immunosorbent assay (ELISA) kits were considered as "golden standard" for comparison. The results show that the quantum dots labeling-based ToRCH microarrays have comparable sensitivity and specificity with ELISA. Besides, the microarrays hold distinct advantages over ELISA test format in detection time, cost, operation and signal stability. Validated by the clinical assay, our quantum dots-based ToRCH microarrays have great potential in the detection of ToRCH-related pathogens.

Keywords Antibody · Diagnosis · Microarray · Quantum dots · Serology

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Introduction

Microarrays (biochips) have been an essential part of the biomarker research workflow for over 10 years. So far, deoxyribonucleic acid (DNA) microarrays are mainly used for gene expression profile analysis and single nucleotide polymorphism (SNP) detection to better classify and detect diseases [1–4]. The protein microarrays are mainly applied to disease-associated serological biomarkers detection, new drug development and biomarker molecules screening. These advantages enable microarrays to become a high-throughput tool applied in the parallel analysis, which can be used to monitor simultaneously the levels of a multitude of target molecules in a single specimen. In many recent reports, the protein microarray approaches with immobilized pathogen-related antigens were used for the parallel detection of relative antibodies in sera [5–8]. Based on the principles of antigen–antibody interaction, almost any combination of pathogen-related antigens with specific purposes can be immobilized onto the surface of microarray substrates for the diagnosis of corresponding diseases. However, for some reasons, the application of this technology into diagnostics and clinical practice has developed very slowly. In clinical diagnosis, there is an increasing need for simple and sensitive analytical method for antigen or antibody detection. One of the solutions is to seek a kind of sensitive and stable labeling substance. Take traditional colloidal gold as an example, it has been

intensively used to label antibodies and antigens for rapid diagnosis of diseases [9, 10], but in fact, the sensitivity of traditional gold nanoparticles-based diagnosis method cannot fully meet the requirement of the rapid and sensitive diagnosis. As nanotechnology advances fast, the nanomaterials bring a new opportunity to the development of simple and sensitive diagnostic tools [11–13].

Quantum dots (QDs), one of those promising nanomaterials, have been subjected to intensive investigations because of their unique photoluminescent properties and potential applications. So far, several methods have been developed to synthesize water-soluble quantum dots for use in biologically relevant studies [14]. According to previous reports, quantum dots have been successfully used in cellular imaging [15], immunoassays [16], DNA hybridization [17] and optical bar-coding [18]. Moreover, quantum dots also have been used to study the interaction between protein molecules or detect the dynamic course of signal transduction in live cells by fluorescence resonance energy transfer (FRET) [19]. These synthesized quantum dots have significant advantages over traditional fluorescent dyes, including better stability, stronger fluorescent intensity and size-tuning colors, which are adjusted by controlling the size of quantum dots [20]. Therefore, quantum dots provide a new functional platform for bioanalytical sciences and biomedical engineering.

In the obstetrics, the ToRCH is referred as five pathogens including Toxoplasmosis, Rubella, Cytomegalovirus and Herpes simplex virus type 1 and 2. If ToRCH-related pathogens infect pregnant women, they will cause severe fetal anomalies or even fetal loss [21, 22]. For this reason, it is of great practical significance to establish a simple, sensitive detection method for ToRCH pathogens. Here, we report a quick and parallel analytical method based on quantum dots labeling for ToRCH-related antibodies. In our work, we fabricated the microarrays with the five ToRCH-related antigens and screened 100 specimens of randomly selected clinical sera from outpatients along with quantum dots-labeled secondary antibody. At the same time, we selected commercially available enzyme-linked immunosorbent assay (ELISA) kits to detect these specimens as "golden standard"; the results were compared with that of quantum dots label-based ToRCH microarrays.

Materials and Methods

Materials

Thioglycolic acid (TGA), sodium borohydride and carbodiimide hydrochloride (EDC) are purchased from Sigma (Sigma, USA) and were used without further treatment or purification. All synthetic preparations and measurements were carried out in Millipore water as solvent with 18 MΩ/cm or less. All other analytically purified reagents were purchased domestically. The nitrocellulose membrane with a pore size of 0.45 μm was purchased from Amersham (GE healthcare, USA). The macromolecular bibulous materials and the outer plastic shells were customized from Yi li Packaging Co Ltd. Human immunoglobulin G (IgG) and sheep-anti-human secondary antibodies, as well as bovine serum albumin (BSA), were purchased from Sigma (Sigma, USA). Five ToRCH-related antigens (Toxoplasmosis, Rubella, Cytomegalovirus and Herpes simplex virus type 1 and 2) were purchased from Microbix Biosystems (Canada). Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4 and 25 mM Na_2HPO_4, pH 7.4, 1% glycerol) and PBST buffers (PBS buffer with 0.05% Tween-20) were used. The ELISA kits for comparison were purchased from Zeus (Zeus, USA).

Collection of Samples

Negative and positive control sera of the five ToRCH antibodies were from Fourth Military Medical University and were kept in our laboratory, all of which were validated jointly by two commercial ELISA kits (Viron and Diesse). The validation assays were performed according to the manufacturer's instructions. About 100 random sera were collected from outpatients at the gynecological department of Tangdu Hospital and frozen at −20 °C. This study was performed according to the Helsinki Declaration and was approved by the Ethical Committee of Fourth Military Medical University.

The Preparation of TGA-Capped QDs

QDs were prepared according to our previous report [13]. The concrete steps are as follows: 5 mM of $CdCl_2{\cdot}2.5\ H_2O$ was dissolved in 110 mL of water, and 12 mM of TGA was added under stirring; this was followed by adjusting the pH to 11 by dropwise addition of 1 M NaOH solution. The solution was placed in a three-necked flask deaerated by N_2 bubbling for 30 min. Under stirring, 2.5 mM of oxygen-free NaHTe solution, which was freshly prepared from tellurium powder and $NaBH_4$ (molar rate of 1:2) in water at 0 °C, was injected into the three-necked flask. The resultant mixtures were refluxed at 100 °C for 4 h. By this means, the 2.5 nm diameter TGA-capped CdTe QDs emitted with a maximum wavelength around 545 nm were obtained.

The Characterization of QDs

Photoluminescence (PL) spectra were used to characterize the fluorescent properties of QDs; they were measured in

1 cm quartz in air at room temperature using a Perkin Elmer LS 55 spectrofluorimeter. The size determination of QDs was performed using a transmission electron microscope (TEM, JEM2010, at 200 kV).

The Conjugation of Sheep-Anti-Human Secondary Antibodies with QDs

After the synthesis of QDs, 50 μL of 2 mg/mL QDs and 400 μL of 2 mg/mL sheep-anti-human secondary antibodies were mixed, and then 300 μL of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide EDC (44 mM in borate buffer) were added and blended by vortex. The resulting solution was allowed to react at room temperature for 1 h with continuous mixing and then at 4 °C for 24 h. The overnight QDs-labeled secondary antibodies were centrifuged at 12,000 rpm for 20 min, and the supernatant was discarded. PBST was used to resuspend and wash QDs-labeled secondary antibodies by centrifugation at 12,000 rpm for three times. Finally, the QDs-labeled secondary antibodies were dispersed in PBST and kept at 4 °C until use.

Preparation of Microarrays

The rationale of QDs-based microarray is shown in Fig. 1. Briefly, the antigens of Toxoplasma gondii, Rubella virus, Cytomegalovirus and Herpes simplex viruses were printed on a nitrocellulose membrane with a pore size of 0.45 μm at a concentration of 1 mg/mL by computer-controlled, high-speed robotics. Each pin of the robotics was estimated to transfer about 1 nL of antigen solution to the nitrocellulose membrane. The microarray consisted of a 4 × 4 matrix, including the five pathogen-related antigens and human IgG positive control in duplicate (Fig. 2). Bovine albumin was printed on the microarray as blanking control indicating possible cross-contamination between pins. Following printing, the microarrays were blocked with PBS containing 10 mg/mL bovine albumin for 1 h at 37 °C and then assembled with macromolecular bibulous materials and plastic outer shells. The finished microarrays were stored at 4 °C until further usage.

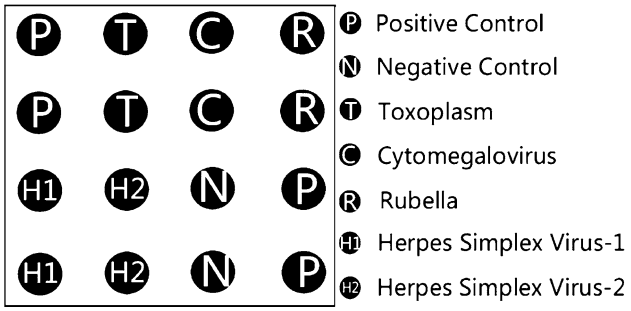

Fig. 2 Schematic representation of microarray

Detection of Clinical Specimens

The procedure for testing with microarray is as follows: firstly, 100 μL of PBST was added onto the microarray to wet it. After PBST infiltrated through the nitrocellulose membrane, 200 μL of serum was added, then the membrane was washed for three times with PBST in order to remove unbound antibodies. About 300 μL of QDs-labeled secondary antibody solution was added subsequently. The membrane was washed three times with PBST again. The portable ultraviolet lamp was used to excite QDs-labeled secondary antibodies on microarray, and the test result was determined by naked eyes. The existence of QDs fluorescence on the spot immobilizing corresponding antigens was determined as positive specimen and vice versa. In our experiment, 10 negative and positive control sera were used to validate ToRCH microarrays. About 100 random sera were tested for ToRCH-related antibodies with our microarrays and commercial ELISA kit from ZEUS Scientific, Inc. The performance of ELISA was based on the manufacturer's instructions. The assay results were analyzed with Statistics Package for Social Sciences (SPSS) software. The sensitivity and specificity of our microarray are calculated by following formulas:

$$\text{Sentivity} = \frac{\text{True positive}}{\text{True positve} + \text{False negative}}$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}.$$

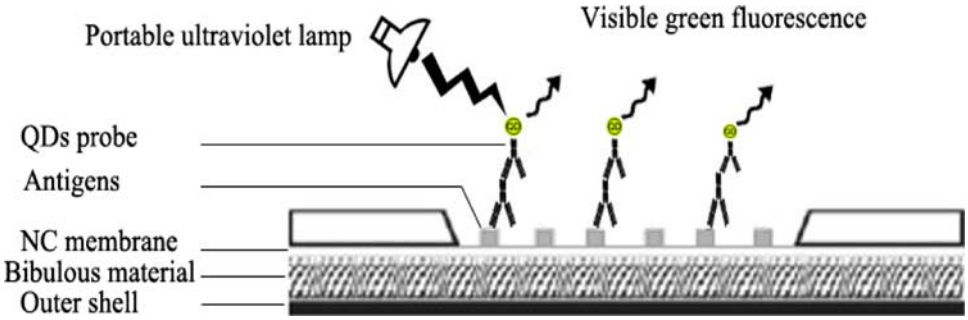

Fig. 1 The rationale of QDs-based microarray

Results and Discussion

The diameter and narrow size distribution of as-prepared CdTe nanocrystals synthesized in this work were determined by TEM. A representative example was presented in Fig. 3. The mean particle diameter is 2.5 nm. The fluorescence spectra and images of QDs before and after the coupling with sheep-anti-human secondary antibodies under UV irradiation are shown in Fig. 4. According to our results, the QDs have strong fluorescence, and the maximum fluorescence wavelength for CdTe was at approximately 545 nm. Besides, green-yellow fluorescence could be observed under a portable UV lamp. However, the blue shift occurred after the coupling of QDs with sheep-anti-human secondary antibodies, and the yellow fluorescence of QDs turned green. The probable reason is that the carboxyl group on the surface of QDs was covalently combined with the amino group of sheep-anti-human IgG, reduced the surface charge of QDs, decreased the polarization rate of the surrounding molecules and then reduced the Stokes displacement, finally resulting in a blue shift in the emission spectra [23]. Meanwhile, the full width at half maximum (FWHM) of the QDs and QDs-labeled antibody remained constant, which meant no aggregation happened to the QDs in the coupling process.

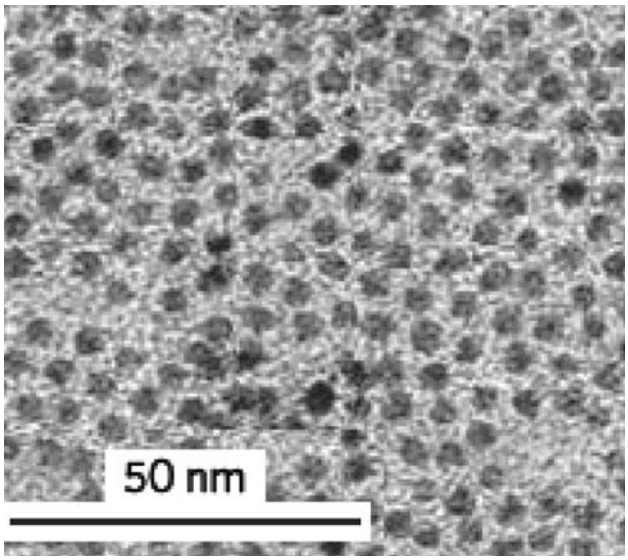

Fig. 3 TEM image of prepared QDs

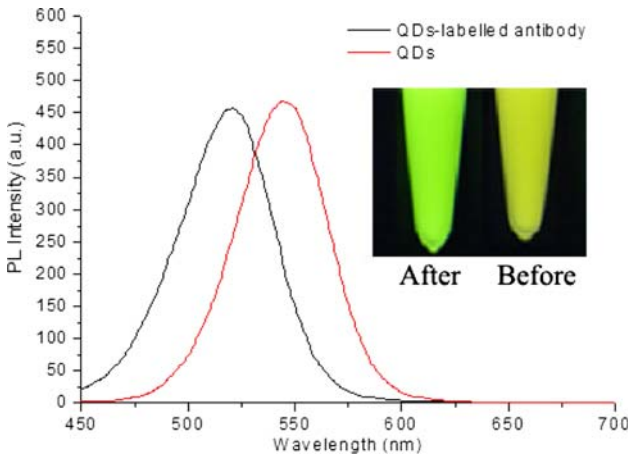

Fig. 4 Fluorescence spectra of QDs solution before and after labeling with goat-anti-human IgG (*inset*: images of QDs before and after the coupling with second antibodies under UV irradiation)

We randomly selected 20 microarrays from as-prepared microarrays and validated them with control sera; the random detection results were consistent with corresponding control sera (Fig. 5). Afterward, 100 random sera from the outpatients were analyzed with our microarrays and the commercial ELISA kits, respectively. The assay results were analyzed using SPSS software. The test results showed that the infectious rates of ToRCH-related agents in these specimens collected from the gynecological department were 83% for Toxoplasmosis, 91% for Rubella, 82% for Cytomegalovirus, 73% for Herpes simplex virus type 1 and 18% for Herpes simplex virus type 2, which were rather close to those of commercial ELISA kits. Moreover, there is no significant difference between the two methods. Compared with the ELISA kits, both sensitivity and specificity of the microarrays exceeded 85% (Table 1).

The prenatal screening of ToRCH-related pathogens is of great significance in eugenics. Up to the present, the ELISA test format has been playing an important role in the first-line method of diagnosis for current, recent or past infection of those ToRCH-related pathogens [24, 25]. The inherent methodological limitation of ELISA test format makes it a time-consuming and tangled laboratory diagnostic format. Especially, during screening for multiple targets from the same analyte, the repetitious procedures multiple the laboratorial workloads undoubtedly. Although there have been many automatic immunowashers that were commercially developed in order to decrease laboratorial workloads and increase test throughput, they still could not resolve the problem of inflexibility and single test time. In this regard, the combination of filtration assay with QDs-labeled probe can offer great advantages over enzyme-based and other kinds of fluorescence-based analytical formats. According to our experiments, compared with ELISA format, the QDs-based microarray possesses these unsurpassable advantages including parallel analysis, test time, test cost, signal stability and instrument requirement. The currently available ELISA and fluorescent immunological test suffer from laborious repeated procedures, while our microarrays allow parallel analysis of multiple target molecules. In addition, each ELISA or fluorescent immunological test will cost a skilled laboratory assistant 2–3.5 h, while our microarray tests only require

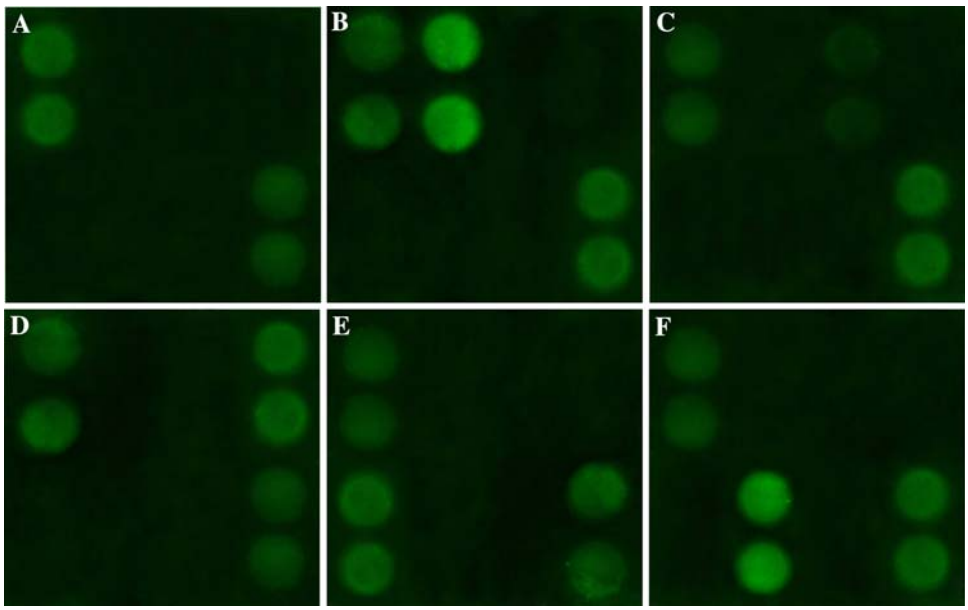

Fig. 5 The microarray results with corresponding control sera (**a** negative serum; **b** positive control serum of Toxoplasmosis; **c** positive control serum of Cytomegalovirus; **d** positive control serum of Rubella virus; **e** positive control serum of Herpes simplex virus type 1; **f** positive control serum of Herpes simplex virus type 2)

Table 1 Comparison of results between QDs-based microarray and ELISA

	ELISA			ELISA			ELISA			ELISA			ELISA		
	TOX +	TOX −	Sum	RV +	RV −	Sum	CMV +	CMV −	Sum	HSV I +	HSV I −	Sum	HSV II +	HSVII −	Sum
+	81	2	83	90	1	91	80	2	82	71	2	73	14	4	18
−	1	16	17	1	8	9	2	16	18	2	25	27	2	80	82
Sum	82	18	100	91	9	100	82	18	100	73	27	100	16	84	100
χ^2			0			0.5			0.25			0.25			0.17
p			<0.05			<0.05			<0.05			<0.05			<0.05

$p < 0.05$, there was no significant difference between the two methods

approximately 10–20 min through four simple steps. Besides, each spot of detection only needs 10–20 ng antigens, whereas ELISA format needs 100–200 ng antigens for coating. Moreover, the signal stability of QDs probe is strong and lasting. As reported previously, dihydrolipoic acid (DHLA)-capped cadmium selenide–zinc sulfide (CdSe–ZnS) QDs showed no loss in intensity after 14 h and were nearly 100 times as stable as, and also 20 times as bright as Rhodamine 6G [19]. Last but not least, the identification of the microarray results does not need any special instrument, whereas the ELISA method needs an enzyme immune analyzer, and other fluorescent immunological tests need an expensive fluorescent scanner or detector.

Conclusions

In this paper, we prepared QDs-based protein microarray to simultaneously detect ToRCH-related antibodies in sera. The rationale of detection is based on immunofiltration assay and QDs-labeled probes. So far, the advent of protein microarrays has made flexible, high-throughput screening of multiple targets in different analytes come true. However, only an easy-to-use and cheap solution is much more suitable for the popularization of this advanced detection format. Our microarrays have shown the unique advantages in aspects of parallelism, cost, signal stability and usability, overcoming most limitations of the current prevalent ELISA test format. Furthermore, compared with other fluorescent immunological microarrays based on glass or silicon chip, our microarrays are much rapider in detection time, much easier in operation, much more stable and stronger in signal. Validated by clinical application, there is no significant difference between the current golden standard ELISA and our microarray in detecting ToRCH infections. Although the results are promising, there is still a need to develop a kind of miniaturized ultraviolet reader to objectively differentiate and sensitively detect clinical specimens in practical application. However, without expensive laser excitation source and confocal scanner, this kind of reader will be much cheaper and portable. In

conclusion, our microarray has a high potential in mass prenatal on-site screening or epidemiological research.

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